Highly Purified Eicosapentaenoic Acid Increases Interleukin-10 Levels of Peripheral Blood Monocytes in Obese Patients With Dyslipidemia

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OBJECTIVE—It has recently been highlighted that proinflammatory (M1) macrophages predominate over anti-inflammatory (M2) macrophages in obesity, thereby contributing to obesity-induced adipose inflammation and insulin resistance. A recent clinical trial revealed that highly purified eicosapentaenoic acid (EPA) reduces the incidence of major coronary events. In this study, we examined the effect of EPA on M1/M2-like phenotypes of peripheral blood monocytes in obese dyslipidemic patients.

RESEARCH DESIGN AND METHODS—Peripheral blood monocytes were prepared from 26 obese patients without and 90 obese patients with dyslipidemia. Of the latter 90 obese patients with dyslipidemia, 82 patients were treated with or without EPA treatment (1.8 g daily) for 3 months.

RESULTS—Monocytes in obese patients with dyslipidemia showed a significantly lower expression of interleukin-10 (IL-10), an M2 marker, than those without dyslipidemia. EPA significantly increased serum IL-10 and EPA levels, the EPA/arachidonic acid (AA) ratio, and monocyte IL-10 expression and decreased the pulse wave velocity (PWV), an index of arterial stiffness, compared with the control group. After EPA treatment, the serum EPA/AA ratio was significantly correlated with monocyte IL-10 expression. Only increases in monocyte IL-10 expression and serum adiponectin were independent determinants of a decreased PWV by EPA. Furthermore, EPA significantly increased the expression and secretion of IL-10 in human monocytic THP-1 cells through a peroxisome proliferator–activated receptor (PPAR)γ-dependent pathway.

CONCLUSIONS—This study is the first to show that EPA increases the monocyte IL-10 expression in parallel with decrease of arterial stiffness, which may contribute to the antiatherogenic effect of EPA in obese dyslipidemic patients.

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The monocyte–macrophage system plays a role in the pathogenesis of obesity and atherosclerotic disease (1,2). This system shows at least two distinct phenotypes of differentiation: proinflammatory (M1) and anti-inflammatory (M2) (3). It has been reported that, in obese adipose tissue, macrophage accumulation is increased, and proinflammatory M1 macrophages predominate over anti-inflammatory M2 macrophages, thereby contributing to obesity-induced adipose inflammation and insulin resistance (4–6). The expression of both M1 and M2 markers is detected in peripheral blood mononuclear cells and even in atherosclerotic plaques (7,8). We and others also provided evidence for the inflammatory state and unfavorable M1/M2-like phenotypes of peripheral blood monocytes in obese diabetic patients (9,10). In particular, interleukin-10 (IL-10), an anti-inflammatory cytokine and M2 marker, might be involved in M2 macrophage recruitment, thus contributing to reducing inflammation and improving the insulin signal (5,11).

In epidemiological and clinical trials, fish oil and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) were found to reduce the incidence of coronary heart disease (12). A large-scale, prospective, randomized clinical trial, the Japan Eicosapentaenoic Acid Lipid Intervention Study (JELIS), demonstrated that highly purified eicosapentaenoic acid (EPA), a specific n-3 PUFA used clinically to treat dyslipidemia, significantly reduces the incidence of major coronary events via cholesterol-independent mechanisms (13). As antiatherogenic effects, we previously demonstrated that EPA reduces atherogenic lipoproteins and C-reactive protein (CRP), an inflammatory marker, as well as the pulse wave velocity (PWV), an index of arterial stiffness, and increases the secretion of adiponectin, the only established adipocytokine with anti-inflammatory and antiatherogenic properties, in obese patients (14–16). We also reported that EPA markedly inhibits LPS-induced monocyte adhesion to the aortic endothelium in parallel with the suppression of endothelial adhesion molecules intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 (17). Several studies showed that dietary n-3 PUFAs and EPA inhibit the ability of macrophages to secrete several effector

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molecules that may be involved in the pathogenesis of atherosclerosis (18,19). Given these protective effects of n-3 PUFAs and EPA on the monocyte–macrophage system (17–19), it is tempting to speculate on the beneficial effect of EPA on the M1/M2-like phenotypes of peripheral blood monocytes in obese patients during the progression of atherosclerosis; however, no direct evidence for such an effect of EPA has been established. EPA can be metabolized to anti-inflammatory eicosanoids and also competitively inhibits the production of arachidonic acid (AA), an n-6 PUFA, and inflammatory eicosanoids derived from AA, which is the precursor of important molecules involved in inflammation and atherosclerotic process (20). Subanalysis of JELIS and other studies suggested that a decreased serum EPA/AA ratio is significantly associated with the incidence of cardiac death and myocardial infarction and the coronary plaque score (21,22).

In this study, we demonstrate for the first time that EPA increased IL-10 RNA expression in peripheral blood monocytes of obese patients with dyslipidemia in parallel with the decrease of arterial stiffness. In addition, the serum EPA/AA ratio after EPA treatment was significantly correlated with IL-10 RNA expression of monocytes. Furthermore, EPA enhanced the expression level of IL-10 RNA through peroxisome proliferator–activated receptor (PPAR)γ in vitro. As EPA reduced the risk of major coronary events in a large-scale, prospective, randomized clinical trial (13), this study provides important insight into its therapeutic implications for obesity-related metabolic sequelae and cardiovascular disease.

RESEARCH DESIGN AND METHODS

Subjects

A total of 116 Japanese obese outpatients were recruited in our clinic during the period from January 2008 to January 2009. Obese patients were defined as those with a BMI of ≥25 kg/m^2, based on the guideline proposed by the Japan Society for the Study of Obesity. Patients with severe renal diseases and severe liver dysfunction were excluded from this study. Patients taking statins, fibrates, angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, insulin-sensitizing agents, or insulin therapy were excluded from the study. All other medications were continued and remained unchanged during the study protocol. This study is a part of the Japan Obesity and Metabolic Syndrome Study, which has undergone clinical trial registration in the University Hospital Medical Information Network (UMIN) system (UMINStudyID: UMIN 000000559) (23). The study protocol was approved by the Ethics Committee for Human Research at Kyoto Medical Center and Tokyo Medical and Dental University. Written informed consent was obtained from all participants.

Study protocol

The study design and enrollment are shown in Supplementary Fig. 1. All of the 116 obese patients who were enrolled were also classified into those with or without dyslipidemia using the following criteria: a triglyceride (TG) level ≥1.69 mmol/L and/or HDL-cholesterol (HDL-C) <1.04 mmol/L for men and <1.29 mmol/L for women, which are some of the components of the criteria for metabolic syndrome proposed by the U.S. National Cholesterol Education Program-Adult Treatment Panel III (24), as described previously (25). Of all 90 obese patients with dyslipidemia enrolled, 8 patients dropped out (5 patients stopped consulting the outpatient clinic, and 3 patients withdrew consent). The remaining 82 patients in whom we could continue observation were assigned to the control and EPA-treated groups (Supplementary Fig. 1). This study was a prospective, randomized, open-label, blinded end point design, employing simple randomization. In the EPA-treated group, an EPA capsule (1.8 g daily) containing highly purified (>98%) EPA ethyl ester (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) was administered for 3 months, as previously described (14–17). No patients in this study had taken part in any of our previous studies. The patients' diet was based on the Japan Atherosclerosis Society Guidelines for the Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases, as previously described (14–17).

Data collection and laboratory methods

At the beginning and end of the study, we measured BMI, systolic blood pressure (SBP) and diastolic blood pressures (DBP), fasting plasma glucose (FPG), HbA_{1c}, serum immunoreactive insulin (IRI), TG, HDL-C, and LDL-cholesterol (LDL-C), adiponectin, and CRP, as previously described (14–17). The value for HbA_{1c} (%) is estimated as a National Glycohemoglobin Standardization Program equivalent value (%) calculated by the formula HbA_{1c} (National Glycohemoglobin Standardization Program) (%) = 1.02 × HbA_{1c} (Japan Diabetes Society) (%) + 0.25% (26). Serum was prepared by collecting blood into glass tubes without an anticoagulant and obtained by centrifugation (1,500 × *g*, 10 min, 4°C), and aliquots of the supernatant were frozen (−80°C) until use. The serum fatty acid levels, such as EPA and AA, were determined by capillary gas chromatography, as described elsewhere (21,22). The serum levels of IL-10 were measured using commercially available immunoassays (R&D Systems, Minneapolis, MN) (27). The mean coefficient of variance for this serum IL-10 ELISA assay shown in its manual was 5.9–7.5%. The brachial-ankle PWV was assessed using the Vasera VS-1000 vascular screening system (Fukuda Denshi, Tokyo, Japan), as described previously (10,16,23). The investigators who performed the vascular measurements were blinded to the patients' characteristics.

Peripheral blood mononuclear cells were collected from heparinized blood samples through density gradient centrifugation with lymphocyte separation solution (Nacalai Tesque, Kyoto, Japan). Human monocytes were obtained by magnetic-assisted cell sorting with anti-human CD14 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the expression of tumor necrosis factor α (TNF-α), IL-6, and IL-10 mRNA was analyzed using a real-time quantitative PCR method (9,10). Details of the primers used are described in Supplementary Materials and Methods. The percentages of $CD163^+CD14^+$ peripheral blood monocytes were analyzed by flow cytometry (FACSCanto; BD Biosciences) with human antibodies against CD14 and CD163 (BD Pharmingen) (10).

Cell culture experiments

Details of cell culture, PPARα and PPARγ silencing by small interfering RNA (siRNA), chromatin immunoprecipitation (ChIP) assays, and transient transfection and luciferase assays are described in Supplementary Materials and Methods.

Statistical analysis

The sample size was calculated with a type I error of 5%, a statistical power of 80%

Table 1—*Clinical characteristics and metabolic variables, M1/M2 markers in peripheral blood monocytes, and PWV before and after treatment with EPA*

	Control		EPA	
	Baseline	After 3 months	Baseline	After 3 months
n (male/female)	26/13		22/21	
Age (years)	54.0 ± 13		52.3 ± 13	
BMI (kg/m^2)	29.1 ± 5.3	29.0 ± 5.5	29.9 ± 4.9	29.6 ± 4.4
SBP (mmHg)	141 ± 19	137 ± 16	139 ± 18	138 ± 18
DBP (mmHg)	83.9 ± 11	83.1 ± 9	83.8 ± 11	85.6 ± 11
EPA (μg/mL)[a]	42 (27–83)	60 (28–88)	51 (31–105)	110 (87–167)**
EPA/AA[a]	0.3 (0.2–0.5)	0.4 (0.2–0.6)	0.3 (0.2–0.7)	0.8 (0.7–1.2)**
FPG (mmol/L)[a]	6.2 (5.3–7.9)	6.1 (5.3–7.6)	6.3 (5.3–7.2)	6.5 (5.4–7.4)
HbA_{1c} (%)	6.5 (5.5–7.3)	6.4 (5.6–7.0)	6.3 (6.0–7.7)	6.2 (5.8–7.4)*
IRI (pmol/L)[a]	62 (36–131)	62 (32–100)	77 (53–136)	77 (53–103)
TG (mmol/L)[a]	2.0 (1.7–2.5)	1.8 (1.4–2.5)	2.3 (1.9–3.2)	1.7 (1.5–2.3)**
HDL-C (mmol/L)	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.3
LDL-C (mmol/)	3.1 ± 0.6	3.0 ± 0.6	3.4 ± 0.8	3.2 ± 0.8
Adiponectin (μg/mL)[a]	6.5 (4.3–8.7)	6.2 (4.3–8.0)	6.1 (4.3–7.2)	6.6 (4.4–8.8)*
CRP (μg/mL)[a]	1.1 (0.5–1.9)	1.0 (0.4–1.6)	0.7 (0.5–2.5)	0.6 (0.4–1.2)
IL-10 (pg/mL)	5.1 ± 1.6	4.9 ± 1.5	5.2 ± 2.1	6.2 ±1.9**
PWV (cm/s)	1,403 ± 221	1,374 ± 206	1,476 ± 298	1,406 ± 296**
Expression in monocytes				
TNF-α (arbitrary units)[a]	1.2 (0.2–3.2)	1.4 (0.2–2.6)	2.3 (1.1–3.3)	2.3 (1.2–3.5)
IL-6 (arbitrary units)[a]	5.1 (2.6–7.6)	5.0 (3.3–7.0)	7.0 (4.6–9.6)	6.8 (4.3–9.6)
IL-10 (arbitrary units)[a]	4.7 (3.1–7.2)	5.4 (2.0–10.5)	5.5 (2.1–13.3)	10.0 (5.0–18.3)**
CD163/CD14 (%)	55.6 ± 12.5	53.4 ± 11.9	55.1 ± 13.4	57.8 ± 14.0
Proportion of				
Diabetes (%)	46.2		44.2	
Hypertension (%)	61.5		55.8	

Data are shown as the mean ± SD or median (interquartile range), unless otherwise noted. [a]Data were nonnormally distributed and analyzed by nonparametric Wilcoxon test. *$P < 0.05$ and **$P < 0.01$ vs. baseline measurement as determined by a two-way repeated-measures ANOVA (control and EPA groups × before and after treatment).

(type II error of 20%), and a standardized effect size of 0.67, so as to demonstrate an EPA-induced change of 1.0 pg/mL of serum IL-10 as being significant using the paired *t* test. Data are presented as the mean ± SD, unless otherwise indicated, and $P < 0.05$ was considered significant. Logarithmic transformation (ln) was used for the variables that were not normally distributed to make their distribution normal.

Student *t* test was used for comparisons of the means between the two groups at the baseline or posttreatment. Categorical variables were evaluated by the χ^2 test. A two-tailed, paired *t* test was applied to evaluate changes from the baseline to posttreatment (14,16). Some of the data were not normally distributed; therefore, in these cases, the nonparametric Wilcoxon test was used instead of the *t* test. Repeated-measures ANOVA was used to assess the effects of EPA on the measured variables. Pearson correlation coefficient was employed to investigate the correlations among M1/M2 markers, PWV, and metabolic parameters at the baseline, between these changes during the EPA treatment, and between the EPA/AA ratio and IL-10 levels after EPA treatment. Changes from the baseline to those at 3 months are abbreviated as Δ. Pearson partial correlation of ΔIL-10 expression in monocytes and ΔPWV with Δmetabolic parameters during EPA treatment was adjusted for the age, sex, and each initial value. ΔIL-10 expression in monocytes was adjusted for the age, sex, and initial IL-10 expression in monocytes, and, in the case of ΔPWV, it was adjusted for the age, sex, and initial values of SBP and PWV. For in vitro studies, post hoc analysis was performed using the Tukey-Kramer test for the comparison among all groups or Dunnett method for the comparison of specific groups. All analyses were performed using Stat View version 5.0 for Windows (SAS Institute Inc., Cary, NC) and SPSS 12.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Baseline characteristics of obese patients with or without dyslipidemia

A summary of the characteristics of the study cohort and a comparison of the obese patients with or without dyslipidemia are shown in Supplementary Table 1. Of all of the 116 obese patients (64 men and 52 women; mean age, 50.9 years), there were 90 (77.6%) obese patients with dyslipidemia. There were no significant differences in age, BMI, SBP, DBP, FPG, HbA_{1c}, IRI, LDL-C, CRP, PWV, and proportion of diabetes and hypertension between those with and without dyslipidemia. The levels of EPA and the EPA/AA ratio tended to be, but not significantly, lower in obese patients with than in those without dyslipidemia (Supplementary Table 1). The serum levels of TG were significantly higher, and those of HDL-C, adiponectin, and IL-10 were significantly lower in obese patients with than in those without dyslipidemia (TG, HDL-C, $P < 0.01$; adiponectin, IL-10, $P < 0.05$). The obese patients with dyslipidemia exhibited significantly lower IL-10 RNA levels in the peripheral blood monocytes than those without dyslipidemia ($P < 0.05$). There were no significant differences in the expression levels of TNF-α, IL-6, and CD163 in peripheral blood monocytes between the two groups (Supplementary Table 1).

Effect of EPA treatment on metabolic syndrome–related variables, M1/M2 markers in peripheral blood monocytes, and PWV

Among 90 obese patients with dyslipidemia enrolled, 8 excluded subjects who dropped out before randomization had a relatively higher BMI and lower age, FPG, HbA_{1c}, and serum IL-10 than the remaining 82 patients. The remaining 82 obese patients with dyslipidemia in whom we could continue observation were randomly assigned to two groups: the EPA-treated group ($n = 43$, 22 men and 21 women; mean BMI, 29.9 ± 4.9 kg/m^2) and the untreated control group ($n = 39$, 26 men and 13 women; mean BMI, 29.1 ± 5.3 kg/m^2). Among the 82 obese patients with dyslipidemia, no participants dropped out of this study. With regard to the control group, all variables

Table 2—*Correlations related to changes in IL-10 in peripheral blood and PWV during treatment with EPA*

	Expression of monocytes			
	ΔIL-10		ΔPWV	
	r	r_p[a]	*r*	r_p[b]
ΔBMI	0.02	−0.01	0.08	0.10
ΔSBP	−0.03	−0.09	0.02	0.08
ΔEPA	0.25*	0.27*	0.06	0.05
ΔHbA_{1c}	−0.20	−0.17	0.05	0.05
ΔTG	−0.13	−0.13	0.19	0.15
ΔHDL-C	0.15	0.21	0.13	0.10
ΔAdiponectin	0.25*	0.22	−0.31**	−0.35**
ΔIL-10	0.01	−0.15	−0.03	0.07
Expression in monocytes				
ΔTNF-α	−0.02	−0.01	−0.07	−0.03
ΔIL-6	0.04	0.14	0.04	−0.12
ΔIL-10	—	—	−0.27*	−0.26*
ΔCD163/CD14	−0.07	−0.03	−0.08	0.07

In 82 obese patients who were assigned to the control group and EPA-treated groups, correlations related to changes in monocyte IL-10 and PWV were examined. Data are Pearson simple and partial correlation coefficients regarding changes during EPA treatment of IL-10 levels in peripheral blood monocytes and of PWV during treatment with EPA. $n = 82$ patients. [a]Value adjusted for age, sex, and initial IL-10 levels in monocytes. [b]Value adjusted for age, sex, initial systolic blood pressure, and initial PWV. *$P < 0.05$ and **$P < 0.01$.

remained unchanged throughout the study (Table 1). Treatment with EPA for 3 months caused a significant reduction of serum levels of HbA_{1c} ($P < 0.05$) and TG ($P < 0.01$) in parallel with a decrease in PWV ($P < 0.01$) and a significant increase of serum levels of EPA, the EPA/AA ratio ($P < 0.01$), and adiponectin ($P < 0.05$) in obese patients, which are consistent with our previous report (14–17). After EPA treatment, the serum level of IL-10 was also significantly increased in obese patients with dyslipidemia ($P < 0.01$). The expression levels for IL-10 RNA were significantly increased by 1.8-fold in peripheral blood monocytes from obese patients with dyslipidemia (from 5.5 to 10.0; $P < 0.05$) (Table 1). However, there were no appreciable changes in the expression levels of TNF-α, IL-6, and CD163 in peripheral blood monocytes after EPA treatment.

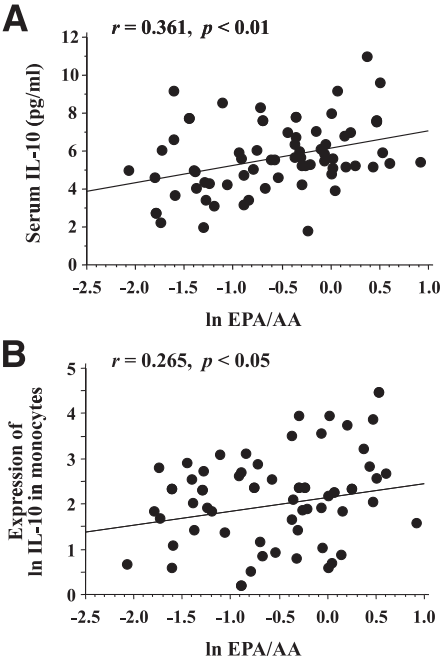

Figure 1—*Association of serum ln EPA/AA ratio with serum level of IL-10* (A) *and expression of ln IL-10 in monocytes* (B) *after 3-month treatment with EPA.*

Correlations related to changes in IL-10 in peripheral blood monocytes and PWV during treatment with EPA

Table 2 lists data for all subjects regarding the correlations observed during EPA treatment between the metabolic syndrome–related variables, IL-10 RNA levels in peripheral blood monocytes, and PWV. In all study subjects, Pearson correlation coefficient revealed that ΔTNF-α, ΔIL-6, and ΔCD163 in peripheral blood monocytes were not correlated with changes in any metabolic variable (data not shown). However, ΔIL-10 expression in peripheral blood monocytes was positively correlated with serum ΔEPA levels and serum Δadiponectin during EPA treatment ($P < 0.05$). Furthermore, ΔPWV by EPA treatment was negatively correlated only with Δadiponectin ($P < 0.01$) and ΔIL-10 expression of monocytes ($P < 0.05$) (Table 2). In addition, after adjusting for age, sex, and initial IL-10, ΔIL-10 expression in peripheral blood monocytes was positively correlated with serum ΔEPA levels during EPA treatment (partial correlation coefficient [r_p] = 0.27; $P < 0.05$). Also, ΔPWV during EPA treatment was negatively correlated only with Δadiponectin ($r_p = -0.35$; $P < 0.01$) and ΔIL-10 expression of monocytes ($r_p = -0.26$; $P < 0.05$) after adjusting for the age, sex, and initial SBP and PWV (Table 2).

Association of serum EPA/AA ratio with serum level of IL-10 and expression of IL-10 in monocytes after EPA treatment

The serum ln EPA/AA ratio after EPA treatment was significantly correlated with the serum IL-10 level and expression level of ln IL-10 in monocytes after EPA treatment (serum IL-10, $r = 0.361$, $P < 0.01$; ln IL-10 expression in monocytes, $r = 0.265$, $P < 0.05$) (Fig. 1*A* and *B*).

Effects of EPA treatment in cultured human monocytic THP-1 cells

To explore the direct effect of EPA on the RNA expression of IL-10, human monocytic THP-1 cells were incubated with 0, 10, or 50 μmol/L EPA for 24 h and stimulated with LPS for 6 h. The expression of IL-10 in THP-1 cells stimulated with LPS was twofold higher than that in THP-1 cells without LPS stimulation ($P < 0.05$) (Fig. 2*A*). Treatment with EPA for 24 h in THP-1 cells with LPS increased the expression of IL-10 in a dose-dependent manner. Treatment with 10 μmol/L EPA significantly increased the expression of IL-10 compared with that without EPA ($P < 0.05$), and treatment with 50 μmol/L EPA significantly increased the expression of IL-10 compared with that without EPA or with 10 μmol/L EPA in THP-1 cells stimulated with LPS ($P < 0.01$) (Fig. 2*A*). We also observed that treatment with 50 μmol/L EPA for 24 h significantly increased the levels of IL-10 protein in medium compared with that without EPA and 10 μmol/L EPA in THP-1 cells stimulated with LPS ($P < 0.01$) (Fig. 2*B*).

Effects of PPAR antagonists on EPA-induced increase of IL-10 expression

We examined whether the effects of EPA on IL-10 expression in THP-1 cells were dependent on PPARα or PPARγ. Incubation of cultures with PPARγ-specific antagonists T0070907 or GW9662 significantly blocked the effect of the treatment with EPA for 24 h on IL-10 expression induced by LPS ($P < 0.05$), whereas the incubation of cultures with the PPARα-specific antagonist MK-886 did not block the effect of EPA on IL-10 expression (Fig. 2*C*). The increased levels of IL-10 protein in medium due to treatment with EPA for 24 h were also blocked with PPARγ-specific antagonists T0070907 and GW9662 ($P < 0.01$), but not with the PPARα-specific antagonist MK-886 (Fig. 2*D*). These findings suggest that EPA exerts anti-inflammatory effects through a PPARγ-dependent pathway.

Effects of EPA on expression of IL-10 in PPAR knockdown THP-1 cells

We used PPARα and PPARγ silencing for further elucidation of the mechanism of action of EPA on the increased expression of IL-10 in THP-1 cells. Treatment with 50 μmol/L EPA, which is within the physiological range observed in study subjects, significantly increased the expression of IL-10 compared with that without EPA ($P < 0.01$). PPARγ knockdown resulted in a loss of EPA-mediated IL-10 production ($P < 0.01$), whereas PPARα knockdown did not alter the effect of EPA on IL-10 expression (Fig. 2*E*). The efficiency of PPARα and PPARγ silencing in THP-1 cells was determined by Western blot analysis, in which decreased expressions of PPARα and PPARγ were observed, respectively. PPARα– and PPARγ–siRNA effectively decreased PPARα and PPARγ levels by ~70% compared with scrambled (nonsilencing) siRNA ($P < 0.05$) (Fig. 2*F*).

Effect of EPA on human IL-10 promoter

In order to examine the effects of EPA on the IL-10 promoter in THP-1 cells, two THP-1 IL-10 promoter luciferase reporter gene constructs, the first containing the IL-10 promoter sequences −421/+120 (pGL3-P421) and the second containing sequences −384/+120 (pGL3-P384), were generated. After 6 h of treatment with LPS (20 ng/mL), the ability of EPA to increase luciferase transcription was assessed. Treatment with EPA (50 μmol/L) significantly ($P = 0.0013$) increased the activity of pGL3-P421 2.4-fold, but did not increase the pGL3-P384 activity (1.2-fold) (Fig. 2*G*). The IL-10 promoter contains peroxisome proliferator response element (PPRE) at −406/−390. These findings indicate that EPA activates the transcription of IL-10, and that IL-10 promoter sequences −421/−384 containing PPRE are required for this activation.

Effect of EPA on binding of PPARγ to IL-10 promoter

To evaluate the influence of EPA on the binding of PPARγ to the endogenous IL-10 promoter in THP-1 cells, ChIP assays were performed. Fig. 2*H* shows the results of quantitative analysis of PCR amplification products before and after the immunoprecipitation of the cross-linked chromatin with anti-PPARγ antibody. These results demonstrate that PPARγ is recruited to the region containing the PPRE site (−406/−390) of the endogenous IL-10 promoter (*lane 5*). In addition, EPA treatment markedly enhanced the PCR amplification product of IL-10, displaying an EPA-induced increase in the binding of PPARγ to the IL-10 promoter (*lane 6*). In contrast, before immunoprecipitation, levels of the IL-10 promoter region containing PPRE were similar between chromatins from cells stimulated with and without EPA (*lanes 1* and *2*, respectively). No PCR-amplified product was found following the immunoprecipitation of the cross-linked chromatin with normal rabbit IgG (*lanes 3* and *4*). PCR amplification with specific primers for the GAPDH gene did not result in significant signals in any immunoprecipitated samples (data not shown), demonstrating the specificity of immunoprecipitation and PCR reactions. These data support the idea that EPA significantly increased the binding of PPARγ to the IL-10 promoter sequences containing PPRE in THP-1 cells.

CONCLUSIONS—In a subanalysis of the JELIS, EPA led to a greater reduction in the risk of coronary artery disease by up to 53% in patients with high TG and low HDL-C levels, two of the risk factors of metabolic syndrome and cardiovascular disease (25). However, this mechanism is still poorly understood.

The current study revealed that levels of IL-10, an anti-inflammatory M2 marker, in monocytes were significantly lower in obese patients with than in those without dyslipidemia. Our data are compatible with a previous report by Esposito et al. (28) showing that serum IL-10 levels were lower in obese women with metabolic syndrome than in those without metabolic syndrome. As IL-10 downregulates the production of proinflammatory cytokines, it is tempting to speculate that the low IL-10 levels observed in obese patients with dyslipidemia represent an attempt to promote continued proinflammatory cytokine production. We also demonstrated for the first time that EPA significantly increases the IL-10 levels in monocytes and serum of obese dyslipidemic patients. These findings indicate that EPA ameliorated the decreased IL-10 RNA levels in monocytes of obese patients with dyslipidemia. This finding is also consistent with the report by Oh et al. (29), demonstrating that fish oil supplementation increases adipose tissue expression of M2 markers, including IL-10, in obese mice. Given the anti-inflammatory and antiatherogenic properties of IL-10 produced by Th2 cytokines (4,11), our results suggest that an EPA-induced improvement of decreased IL-10 RNA levels in monocytes of obese dyslipidemic patients might lead to greater cardioprotective effects of EPA.

In this study, we also confirmed that 3-month treatment with EPA effectively improved PWV, an index of arterial stiffness, as we previously reported (16). Pearson partial correlation in this study revealed that only increased monocyte IL-10 RNA and serum adiponectin levels were significantly correlated with the reduction of PWV by EPA treatment. Recently, it was indicated that cell heterogeneity (M1/M2-like phenotype, especially an increased M1/M2 ratio) of circulating monocytes may influence their ability to attach to endothelial cells and increase plaque formation of atherosclerotic lesions (7,8). IL-10 has also been reported to promote the differentiation of M2 macrophages and attenuate the M1 macrophage population (30). Further, it was reported that the increased IL-10 signaling elicits the differentiation or recruitment of alternative M2 macrophages in adipose tissue in mice, thus contributing to reducing inflammation and improving insulin signaling (11). Previous studies demonstrated that IL-10–transgenic mice or gene transfer of IL-10 reduced atherosclerosis in C57BL/6J mice or murine atherosclerotic models (31,32), whereas their IL-10–deficient counterparts exhibited increased early

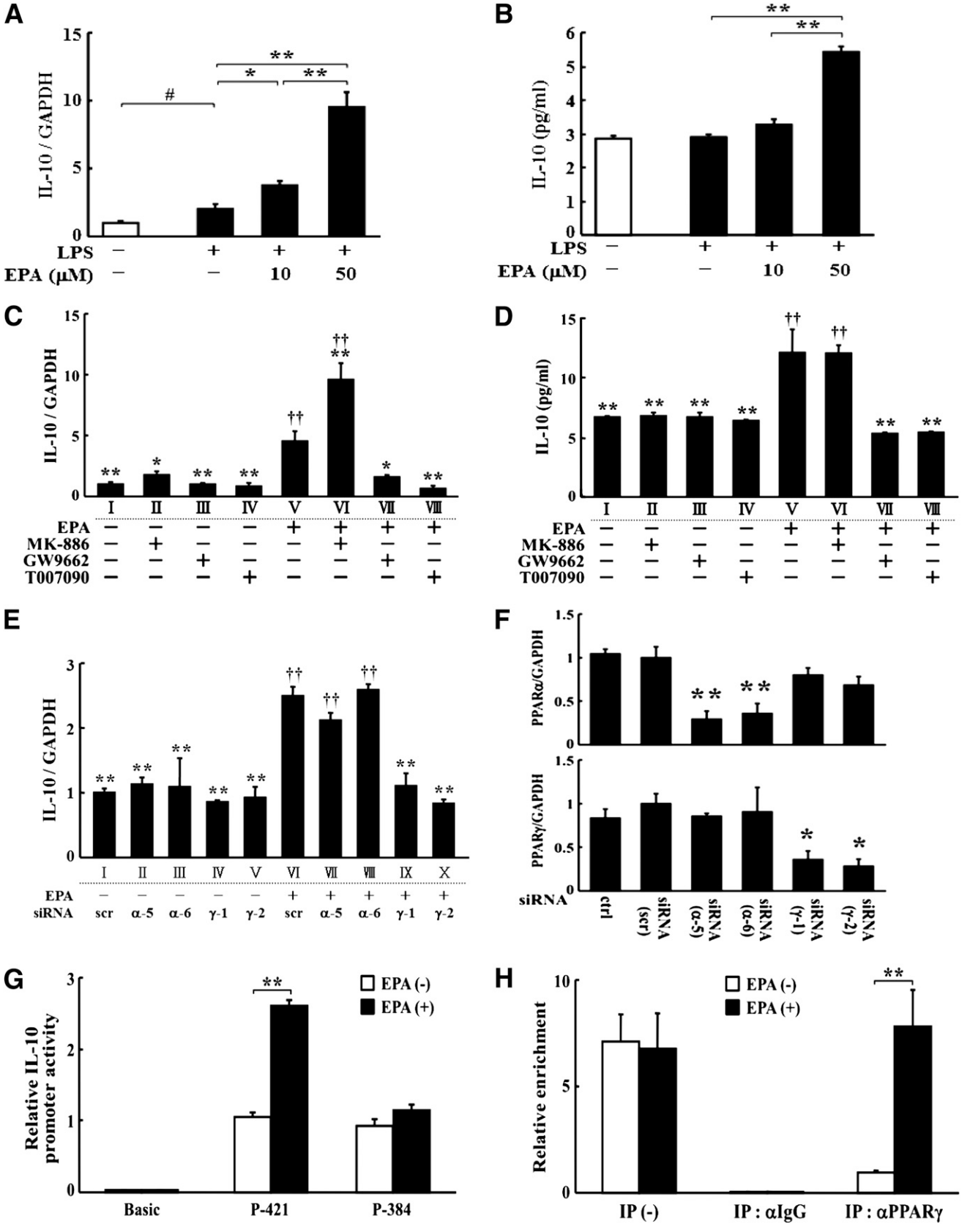

Figure 2—*Effects of EPA treatment in cultured human THP-1 cells.* A *and* B: *Effects of EPA treatment on IL-10 mRNA and protein levels in medium of cultured THP-1 cells. Data are the mean ± SE. Differences between the groups treated with and without LPS within EPA-untreated cells were assessed by Student* t *test. Differences between groups within LPS-treated THP-1 cells were assessed using the Tukey-Kramer post hoc test* (n = 6–8). *IL-10 mRNA expression* (A) *and IL-10 protein levels in medium* (B) *in THP-1 monocytes after treatment without or with 10 or 50 μmol/L EPA for*

atherosclerotic lesion formation (31). In addition, IL-10 overexpression in macrophages inhibited atherosclerosis in LDL receptor-deficient mice (33). Taken together, these findings provide evidence that IL-10 is an important key factor for the prevention of atherosclerosis in vivo. The effect of EPA on IL-10 of monocytes might be attributable to the beneficial effect on arterial stiffness of EPA. However, further examination is necessary to elucidate the causal relationship between an increase of IL-10 expression and the improvement in vascular function by EPA.

Even excluding the influence of age, sex, and the initial value of IL-10 expression in monocytes, only the ΔEPA level was significantly positively correlated with ΔIL-10 expression in monocytes during EPA treatment. In human monocytic THP-1 cells, we found that EPA increased the mRNA expression and protein levels of IL-10 in the medium. In this study, in vitro analysis using PPAR antagonists and siRNA revealed that EPA increased the expression of IL-10 through PPARγ, but not PPARα, in THP-1 cells. This finding is consistent with a previous report showing that EPA decreased IL-6 production through PPARγ, not through PPARα, in C6 glioma cells (34). We have also performed ChIP and luciferase assays to elucidate how EPA regulates IL-10 expression through a PPARγ-dependent pathway. Luciferase assays showed that EPA activates the transcription of IL-10 and that IL-10 promoter sequences containing PPRE are required. In addition, ChIP assays revealed that EPA significantly increased the binding of PPARγ to the IL-10 promoter sequences containing PPRE in THP-1 cells. These results are similar to the findings of a previous study using rosiglitazone, one of the PPARγ ligands (35). From these findings, it is conceivable that EPA increases the binding of PPARγ to the human IL-10 promoter region and activates transcription of the human IL-10 gene. As reported previously, EPA, via the activation of PPARγ, mediates various actions such as the inhibition of IL-6 production in C6 glioma cells and of MMP expression in macrophages and the increase of adiponectin expression in adipocytes (34,36,37). Moreover, PPARγ expression itself has been reported to be significantly associated with increased M2 monocytes in the vasculature and maturation of alternatively activated macrophages (6,8). We also observed that pioglitazone, a PPARγ agonist, increased IL-10 expression and improved the unbalanced M1/M2-like phenotype of monocytes in obese diabetic patients, which may contribute to its antiatherogenic effect (10). Therefore, our findings indicate that the upregulation of IL-10 induced by EPA is potentially mediated in part through the activation of PPARγ. However, further studies on the direct interaction between EPA and PPARγ, such as identification of the binding site using X-ray analysis, are required to clarify whether EPA is a functional ligand of PPARγ.

We revealed that the EPA-induced increase of adiponectin was also significantly negatively correlated with the improvement of PWV through EPA treatment. We previously demonstrated that EPA increases adiponectin in obese mice and obese human subjects, possibly through improvement of the inflammatory changes in obese adipose tissue (15). Previous epidemiological studies reported a significant positive correlation between serum adiponectin and IL-10 in healthy and obese subjects (27). In human monocyte-derived macrophages, adiponectin significantly increased the expression and secretion of IL-10 (38). Recently, Ohashi et al. (39) reported that adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype, which may be partly linked to the decrease of arterial stiffness in our study. Collectively, it is conceivable that EPA increases IL-10 RNA expression of monocytes in hyperinsulinemic obese patients, in part through the enhancement of adiponectin by EPA, thereby additively suppressing the atherogenic process.

The current study showed that EPA treatment significantly increased the serum EPA/AA ratio. In addition, the EPA/AA ratio after EPA treatment was significantly correlated with IL-10 in monocytes. Recently, it was reported that a lower level of EPA was an independent predictor of all-cause-mortality in patients with acute myocardial infarction (40). EPA can be metabolized to anti-inflammatory eicosanoids and also partially replace the AA in cell membranes and competitively inhibits the production of AA and inflammatory eicosanoids, thereby exerting an anti-inflammatory effect (20). There is also a recent report showing that the serum EPA levels and

24 h and subsequent stimulation with LPS for 6 h. The mRNA levels of IL-10 (A) *were measured using quantitative real-time PCR and standardized for the GAPDH levels.* #$P < 0.05$ *by Student* t *test;* *$P < 0.05$, **$P < 0.01$ *using the Tukey-Kramer method.* C *and* D*: Effect of PPARα and PPARγ antagonists on IL-10 mRNA expression and IL-10 protein levels in cultured THP-1 cells. THP-1 monocytes with a density of* 1×10^6 *cells/well were treated with LPS for 6 h in the absence or presence of 50 μmol/L EPA for 24 h. Some cultures were incubated with the PPARα antagonist MK-886 (II, VI), and some with the PPARγ agonists GW9662 (III, VII) or T0070907 (IV, VIII), at a dose of 10 μmol/L in the presence of LPS. The expressions of IL-10 mRNA in THP-1 cells* (C) *and IL-10 protein levels in medium* (D) *were compared with those of cultures incubated without PPAR antagonists in the absence (I) or presence (V) of 50 μmol/L EPA.* ††$P < 0.01$ *vs. group I;* *$P < 0.05$, **$P < 0.01$ *vs. group V using the Dunnett method* (n = 4–6). E *and* F*: Effects of EPA on IL-10 expression by PPAR knockdown in THP-1 cells. Incubation was conducted with scrambled siRNA (scr), PPARα–siRNA (α-5, α-6), or PPARγ–siRNA (γ-1, γ-2) during THP-1 differentiation into macrophages, as described in* RESEARCH DESIGN AND METHODS. *After treatment with siRNA (25 nmol/L), EPA (50 μmol/L), and LPS, cells were harvested, and the mRNA levels of IL-10 were measured by real-time PCR* (E). *The protein levels of PPARα and PPARγ were measured using Western blot analysis, and quantitative data are expressed as the fold of the control scrambled siRNA and are the mean ± SE* (F). *Expression levels were standardized for GAPDH levels. The results of three separately performed experiments are expressed relative to the control and presented as the mean ± SE.* ††$P < 0.01$, *vs. group I;* **$P < 0.01$ *vs. group VI by the Dunnett method* (E). *$P < 0.05$, **$P < 0.01$ *vs. scr by the Dunnett method* (F). G *and* H*: Effects of EPA on human IL-10 promoter and on binding of PPARγ to IL-10 promoter.* G*: Luciferase reporter assays were performed using the luciferase reporter constructs for the human IL-10 promoter. Cells were transiently transfected with either pGL3-P421 (P-421) or pGL3-P384 (P-384) and the control plasmid pRL-TK. After 24 h, cells were treated with EPA (50 μmol/L) for 24 h and were stimulated by LPS (20 ng/mL) for 6 h. Luciferase activity was measured using a luminometer, and the results were normalized against the* Renilla *luciferase control.* **$P < 0.01$ *vs. cells treated without EPA using Student* t *test.* H*: IL-10 promoter ChIP assays were performed using chromatin extracts prepared from THP-1 monocytes treated with or without EPA (50 μmol/L) for 24 h and were stimulated by LPS (20 ng/mL) for 6 h. Control PCRs were carried out with nonimmunoprecipitated genomic DNA [input: IP(-)]. ctrl, control; αIgG indicates anti-rabbit IgG; αPPARγ, anti-PPARγ antibody.* **$P < 0.01$ *vs. cells treated without EPA using Student* t *test.*

AA/EPA ratio were significantly associated with the extent of coronary soft plaques and calcification (21). Furthermore, subanalysis of the JELIS has shown that the EPA/AA ratio is markedly correlated with a lower incidence of sudden cardiac death and myocardial infarction (22). Because EPA competitively inhibits inflammatory eicosanoids produced by AA (20), these findings raise the possibility that the EPA-induced increase of IL-10 RNA levels in monocytes is at least partially caused by inhibition of the proinflammatory effects of AA by EPA.

There are some limitations regarding the current study. Because Pearson correlation coefficients in this study are relatively weak, it is necessary to conduct a long-term prospective cohort study with a larger sample size in order to clarify the involvement of monocyte IL-10 in the improvement of vascular function by the treatment with EPA. Several lines of evidence suggest the important role of monocyte heterogeneity for the local macrophage conditions and inflammation in atherosclerotic lesions in mouse (7,8). However, further investigation is needed to elucidate whether the M1/M2-like phenotype or a higher IL-10 level of circulating monocytes reflects the M1/M2 macrophages and contributes to disease progression in atherosclerotic lesions in human.

In conclusion, this study demonstrates that EPA increases the IL-10 expression in peripheral blood monocytes in parallel with the decrease of arterial stiffness in obese patients with dyslipidemia. Given the anti-inflammatory and antiatherogenic properties of IL-10 (4,11), the beneficial antiatherogenic effect of EPA may be due, at least in part, to increased IL-10 expression and secretion in monocytes. As EPA is a specific n-3 PUFA that has been proven to reduce the risk of major coronary events (13), the results of this study provide important insights into its therapeutic implications in obesity-related atherosclerotic diseases.

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N.S.-A. researched the data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. A.S. contributed to the discussion and reviewed and edited the manuscript. Y.S. researched the data, contributed to the discussion, and reviewed and edited the manuscript. H.N., A.H., and M.T. researched the data. S.K. and H.W. contributed to the discussion. T.T. researched the data. K.O. researched the data and contributed to the discussion. T.S. and Y.O. contributed to the discussion and reviewed and edited the manuscript. K.H. contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. N.S.-A. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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